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(54) Title: KERATINOCYTE OR EPITHELIAL CELL LINE WHICH EXPRESSES HUMAN PAPILLOMAVIRUS E5 GENE

(57) Abstract

A keratinocyte or epithelial cell line that expresses human papillomavirus E5 gene. Such cell line is useful as a drug screen to identify compounds that inhibit the action of the E5 gene.

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KERATINOCYTE OR EPITHELIAL CELL LINE WHICH EXPRESSES HUMAN PAPILLOMAVIRUS E5 GENE

GOVERNMENT RIGHTS

The invention was made with United States government support under grants CA 37157 and CA 16038 from the National Institute of Health. The United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns a keratinocyte or epithelial cell line that expresses human papillomavirus E5 gene.

Background Information

The papillomaviruses are small DNA viruses that induce tumor formation in their natural hosts including humans (Pfister, H., (1984) "The Biochemistry of Papillomaviruses", Rev. Physiol. Biochem. Pharmacol., 99, 111-181). The true papillomaviruses, such as human papillomavirus type 16 (HPV16), induce the formation of papillomas consisting exclusively of epithelial cells. In contrast, the fibropapillomaviruses, such as bovine papillomavirus type 1 (BPV), induce the formation of skin fibropapillomas consisting of proliferating dermal fibroblasts, as well as epidermal keratinocytes. The fibropapillomaviruses also differ from the true papillomaviruses in their ability to efficiently transform rodent fibroblast cell lines in culture. Although the human papillomaviruses can also transform such

cells, this activity is much weaker than that exhibited by the fibropapillomaviruses.

The ability of BPV to transform fibroblasts in vitro is due in large part to the viral E5 protein, which is well conserved among the various fibropapillomaviruses (DiMaio, D., Guralski, D. and Schiller, J. T., (1986), "Translation of Open Reading Frame E5 of Bovine Papillomavirus is Required for its Transforming Activity", Proc. Natl. Acad. Sci. U.S.A., 83, 1797-1801; Horwitz, B.H., Burkhardt, A.L., Schlegel, R. and DiMaio, D., (1988), "44 Amino acid E5 Transforming Protein of Bovine Papillomavirus Type 1 Requires a Hydrophobic Core and Specific Carboxyl-Terminal Amino Acids", Mol. Cell Biol., 8, 4071-4078; Moreno-Lopez, J., Ahola, H., Eriksson, A., Bergman, P. and Pettersson, U., (1987), "Reindeer Papillomavirus Transforming Properties Correlate with a Highly Conserved E5 Region", J. Virol., 61, 3394-3400; Schiller, J.T., Vass, W.C., Vousden, K. and Lowy, D.R., (1986), "The E5 Open Reading Frame of Bovine Papillomavirus Encodes a Transforming Gene", J. Virol. 57, 1-6 and Yang, Y.C., Spalholz, B., Rabson, M., and Howley, P.M., (1985), "Dissociation of Transforming and Trans-activation Functions for Bovine Papillomavirus Type 1", Nature (London), 318, 575-577).

The BPV E5 protein, a very hydrophobic, 44 amino acid membrane-associated protein, is the shortest transforming protein known (Burkhardt, A.L., DiMaio, D. and Schlegel, R., (1987), "Genetic and Biochemical Definition of the Bovine Papillomavirus

E5 Transforming Protein", EMBO J., 6, 2381-2385 and Schlegel, R., Wade-Glass, M., Rabson, M.S. and Yang, Y.C., (1986), "The E5 Transforming Gene of Bovine Papillomavirus Encodes a Small Hydrophobic Protein", Science, 233, 464-467). It has recently been shown that the BPV E5 protein activates the cellular platelet-derived growth factor (PDGF) ß receptor and that there is amino acid sequence similarity between the active site of the BPV E5 protein and PDGF (Petti, L., Nilson, L. and DiMaio, D., (1991), "Activation of the Platelet - derived Growth Factor Receptor by the BPV E5 Transforming Protein", EMBO J., 10, 845-855). On the basis of these results, it is considered that the PDGF receptor is an important cellular mediator of the fibroblast transforming activity of the fibropapillomavirus E5 proteins. Although transfected BPV DNA affects the differentiation of murine keratinocytes (Reiss, M., DiMaio, D. and Zibello, T.A, (1989), "Bovine Papillomavirus Type I. Induces Resistance to Ca^{++}-Induced Terminal Differentiation in Murine Keratinocytes", Cancer Communications, 1, 75-82), the activity of specific BPV genes in keratinocytes has not been determined.

HPV 16 and related viruses can immortalize and affect the differentiation of primary human keratinocytes, an activity that has been mapped to their E6 and E7 genes. Although the E6 and E7 genes are in general not sufficient to confer tumorigenicity upon cultured human keratinocytes, their frequent retention and expression in the human carcinomas associated with HPV16 and 18

infection suggest these genes may play a role in keratinocyte transformation in vivo (zur Hausen, H., and Schneider, A., (1987), "The Role of Papillomaviruses in Human Anogenital Cancers" In, The Papillomaviruses, N.P. Salzman and P. M. Howley, Editors, Plenum Press, pp. 245-292). In addition to the E6 and E7 proteins, many of the HPVs are predicted to encode short, hydrophobic E5 proteins that are only distantly related to the fibropapillomavirus E5 proteins and bear no sequence similarity to PDGF (Bubb, V., McCance, D.J. and Schlegel, R., (1988), "DNA Sequence of the HPV-16 E5 ORF and the Structural Conservation of its Encoded Protein", Virol., 163, 243-246 and Halbert, C.L. and Galloway, D.A., (1988), "Identification of the E5 Open Reading Frame of Human Papillomavirus Type 16", J. Virol, 62, 1071-1075).

Although the ability of HPV16 and HPV18 to transform cultured rodent fibroblasts has been mapped to the E7 gene (DiMaio, D., (1991), "Transforming Activity of Bovine and Human Papillomavirusess in Cultured Cells", Adv. Cancer Research, 56, 133-159), these viruses display residual fibroblast transforming activity when the E7 gene is mutated (Bedell, M.A., Jones, K.A., Grossman, S.R., and Laimins, L.A., (1989), "Identification of Human Papillomavirus Type 18 Transforming Genes in Immortalized and Primary Cells", J. Virol., 63, 1247-1255 and Vousden, K.H., Doniger, J., DiPaolo, J.A. and Lowy, D.R. (1988), "The E7 Open Reading Frame of Human Papillomavirus Type 16 Encodes a Transforming Gene," Oncogene Research, 3, 167-175). It has been

suggested that this activity is due to the E5 gene, but the involvement of the HPV 16 and HPV18 E5 genes in cell transformation has not been directly established. The HPV6c E5a gene has been reported to transform mouse fibroblasts in culture (Chen, S-L. and Mounts, P., (1990), "Transforming Activity of E5a Protein of HBV type 6 in NIH3T3 and C127 Cells", J. Virology, 64, 3226-3233). However, since HPV do not normally infect fibroblasts, the significance of this finding is uncertain.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a cell line that expresses human papillomavirus E5 gene.

It is a further object of the present invention to provide a screen to identify compounds which inhibit E5 gene action.

The above objects, as well as other objects, aimS and advantages are provided by the present invention.

The present invention concerns a keratinocyte or epithelial cell line that expresses a human papillomavirus E5 gene.

The present invention also concerns a drug screening method (to determine if a drug interferes with a property of cells that is due to the expression of the E5 protein) comprising administering to an animal, e.g., by subcutaneous injection or by skin grafting, an effective amount the aforesaid cell line that expresses human papillomavirus E5 gene and then administering an effective amount of a drug to said animal, e.g., by injection, e.g., substaneous injection, or orally to determine if said drug interferes with the ability of the the cells to display a property, e.g., tumor formation, that is due to the expression of the E5 protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A to 1F are schematic diagrams of maps of the recombinant retroviruses used in these experiments. Fig. 1A shows the structure of the original vector, pLXSN (Miller, A. D. and Rosman, G.J., (1989), "Improved Retroviral Vectors for Gene

Transfer and Expression", Biotechniques, 7, 980-990., which was converted into pLXSN-Sal by insertion of a unique Sal I site at the position of the short vertical line. Genes inserted at this site are transcribed from left to right from the promoter in the left LTR. The gene encoding resistance to G418 is transcribed from the SV40 early promoter. The bacterial plasmid vector sequences joining the two LTRs are not shown. Figs 1B to 1F represent the inserted BPV and HPV16 E5 genes, represented by the dark and stippled bars, respectively. The extent of each insert is indicated by the nucleotide numbers of the parental papillomavirus genomes. L1M-FS contains a frameshift mutation in the BPV E5 gene; L1D contains a dimer insert.

Fig. 2A and 2B are photographs of an autoradiograph of a filter showing E5 RNA in mouse fibroblasts. Total RNA was prepared from normal cells or G418 resistant cell lines established from individual, independent drug resistant colonies induced by infection with retrovirus stocks. 10 μg of each sample was electrophoresed in a 1 % agarose gel containing formaldehyde, transferred to nitrocellulose, and hybridized to a probe uniformly labelled with ^{32}P by using random priming of purified restriction fragments. Bands were visualized by autoradiography. The positions of 28S (4.9) and 18S (1.9) ribosomal RNA are indicated.

Fig. 2A: C127: normal C 127 cells; R7-3: cell line isolated following infection with control virus RV-LXSN-Sal; A2, A4, A5, and C1: cell lines expressing BPV E5 gene isolated following infection with RV-BE5-L3. The probe was BPV nt # 3881 to 4450.

Fig. 2B: NIH3T3: normal NIH3T3 cells; R7-3: lanes 1-3, cell lines isolated following infection with the control virus, RV-LXSN-Sal; H16-1: lanes 1-6, cell lines expressing HPV 16 E5 gene isolated following infection with RV-H16E5-1; H16-6, lanes 1-6, cell lines expressing HPV 16 gene isolated following infection with RV-H16E5-6. The probe was HPV16 nt # 3759 to 4151.

Figs. 3A and 3B are photographs of an autoradiograph of a filter showing BPV and HPV E5 RNA in mouse keratinocytes. Total RNA was analyzed as described above with respect to Fig. 2.

P117: normal cells; R7: pooled G418 resistant p117 cells established following infection with control virus, RV-LXSN-Sal; BP1 and BP2: two independent pools of G418 resistant p117 cells transformed with RV-BE5-L3 containing BPV E5 gene; H1·5: pooled G418 resistant p117 cells infected with RV-H16E5-1 containing HPV 16 E5 gene. H6·2:pooled G418 resistant p117 cells infected with RV-H16E5·6 containing the HPV16 E5 gene. The probe was BPV nt # 3881 to 4450 (Fig. 3A) or HPV16 nt # 3769 to 4151 (Fig. 3B). Rehybridization of the filters with an actin probe demonstrated

that all samples from G418-resistant cell lines contained similar amounts of intact RNA (data not shown).

Figs. 4A-4C are photomicrographs of stained tumor sections depicting the histologic appearance of tumors formed by p117 cells expressing the E5 genes.

Fig 4A. (HBV16 E5 tumor): Note the squamous differentiation with presence of basal and spinous layers (x200).

Fig 4B. (HBV16 E5 tumor): Note scattered horn pearls and moderate cytological atypia (x400).

Fig 4C. (BPV E5 tumor): Note the tumor cells growing in between muscle fibers and the presence of numerous mitotic figures (x200).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a keratinocyte or epithelial cell line that expresses human papillomavirus E5 gene.

The following types of human papillomaviruses are discussed in "Association of Human Papillomavirus with Benign, Premalignant and Malignant Anogenital Lesions" by A.B. Jenson and W.D. Lancaster. In Papillomaviruses and Human Cancer by H. Pfister, CRL Press, (1990):

Clinicopathological Grouping of Human Papillomaviruses and Malignant Potential of the Lesions with which They Are Most Frequently Associated

Group I – Cutaneotropic (immunocompetent host)	
1,4	Plantar warts (benign)
2, 26, 28, 29	Common warts (benign)
3, 10, 27	Flat warts (benign)
7	Butcher's warts (benign)
Group 2 – Cutaneotropic (epidermodysplasia verruciformis; immunocompromised host)	
5, 8	Macular lesions (highly malignant)
9, 12, 14, 15, 17 19–25, 36, 46–50	Macular or flat lesions (benign/rarely malignant)
Group 3 – Mucosotropic	
6, 11, 34, 39 41–44, 51–55	Condylomata (rarely malignant)
13, 32	Focal epithelial hyperplasia (benign)
16, 18	Condylomata (highly malignant); bowenoid papulosis
30, 31, 33, 35, 45, 56	Condylomata (intermediately malignant)

Preferred types of E5 gene for use in the present invention include the following: type 1, type 2, type 6, type 11 and type 18, preferably type 16.

A starting retrovirus vector that can be used in the present invention is pLXSN (source: Fred Hutchinson Cancer Research Center). Applicants modified this vector by insertion of a Sal I linker at the unique Xho I site to generate pLXSN-Sal. Other retroviral vectors which can successfully introduce genes into p117 cells include MD1, RasZip6, v-neu, v-53, Mp53 and pK (Dotto et al, Molecular Carcinogenesis, 1, 171-179, (1988)).

HPV16DNA used in the examples reported herein was obtained from the German Cancer Research Center, Heidelberg, Germany. The E5 gene was amplified by using the polymerase chain reaction, and was subcloned into the unique Sal I site of pLSXN-Sal. It is also possible to construct the gene using synthetic oligonucleotides corresponding to the published sequence of the E5 gene (Bubb, V. et al, Virol., 163, 243-247, (1988) and Halbert et al, J. Virol. 62, 1071-1075,(1988)).

The sequence for a HPV 16 E5 gene which can be inserted into an expression vector for use in the present invention is as follows:

SEQ. I.D. NO. 1

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AGTGAATGGC AACGTGACCA ATTTTTGTCT CAAGTTAAAA TACCAAAAAC TATTACAGTG   60

TCTACTGGAT TTATGTCTAT ATG ACA AAT CTT GAT ACT GCA TCC ACA ACA      110
                     Met Thr Asn Leu Asp Thr Ala Ser Thr Thr
                      1               5                  10

TTA CTG GCG TGC TTT TTG CTT TGC TTT TGT GTG CTT TTG TGT GTC TGC    158
Leu Leu Ala Cys Phe Leu Leu Cys Phe Cys Val Leu Leu Cys Val Cys
                 15                  20                  25

CTA TTA ATA CGT CCG CTG CTT TTG TCT GTG TCT ACA TAC ACA TCA TTA    206
Leu Leu Ile Arg Pro Leu Leu Leu Ser Val Ser Thr Tyr Thr Ser Leu
             30                  35                  40

ATA ATA TTG GTA TTA CTA TTG TGG ATA ACA GCA GCC TCT GCG TTT AGG    254
Ile Ile Leu Val Leu Leu Leu Trp Ile Thr Ala Ala Ser Ala Phe Arg
         45                  50                  55

TGT TTT ATT GTA TAT ATT ATA TTT GTT TAT ATA CCA TTA TTT TTA ATA    302
Cys Phe Ile Val Tyr Ile Ile Phe Val Tyr Ile Pro Leu Phe Leu Ile
     60                  65                  70

CAT ACA CAT GCA CGC TTT TTA ATT ACA TAATGTATAT GTACATAATG          349
His Thr His Ala Arg Phe Leu Ile Thr
 75                  80

TAATTGTTAC ATATAATTGT TGTATACCAT AACT                              383
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A portion of the above sequence including the intact coding region can also be inserted into a vector.

The gene can be introduced into cells via retrovirus infection. The "LIPOFECTION" technique can also be used. The "LIPOFECTION" technique is a liposome-mediated transfection protocol for the introduction of DNA into animal cells (Philip L. Felgner, Thomas R. Gadek, Marilyn Holm, Richard Roman, Hardy W. Chan, Michael Wenz, Jeffrey P. Northrop, Gordon M.Ringold an Mark Danielsen, "Lipofection: A Highly Efficient, Lipid-Mediated DNA-Transfection Procedure", Proc. Natl. Acad. Sci. USA, 84, 7413-7417 (1987)). This protocol uses the synthetic cationic lipid DOTMA (N-[1-(2,3-dioleyloxy)- propyl]-N,N,N-trimethyl-ammonium chloride). Liposomes composed of DOTMA and a neutral lipid PtdEtn (dioleoylphosphatidylethanolamine) form stable complexes with DNA, and deliver DNA into several eukaryotic cells

with higher efficiency and reproducibility than some oher methods.

Other techniques that have also been used to introduce DNA into keratinocytes, and which can be employed in the present invention are electroporation and calcium phosphate-mediated transfection.

The cell keratinocyte or epithelial cell line can be from an animal species such as mouse, rat, rabbit, pig or goat. Non-limiting examples of particular cell lines for use in the present invention are murine kerotinocyte cell line p117 and murine keratinocyte cell line Pam 212.

The present invention is based on the discovery that HPV16 and BPV E5 genes can transform keratinocytes to tumorigenicity. The HPV16 E5 gene, which has not been previously identified as a transforming gene, now emerges as a possible contributor to the neoplastic proliferation induced by HPV16 in vivo. Applicants were the first to demonstrate that HPV E5 expression can affect keratinocyte proliferation and differentiation. Thus is it considered that HPV E5 expression will play a role in human diseases caused by these viruses including warts (papillomas), cervical dysplasia and possibly carcinomas. If E5 in fact plays a role in these diseases, drugs that counteract E5 action will be therapeutically useful.

p117 cells have proven useful in assessing the differential effects of various oncogenes in skin carcinogenesis in vivo (Dotto, G.P., O'Connell, J., Patskan, G. Conti, C., Ariza, A., and Slaga, T. J., (1988), "Malignant Progression of Papilloma-

Derived Keratinocytes: Different Effects of the ras, neu, and p53 Oncogenes", Molecular Carcinogenesis, 1, 171-179). Although these cells were derived form a benign, chemically- induced skin papilloma in a Sencar mouse, they are consistently non-tumorigenic upon subcutaneous injection into nude mice (Dotto et al, Molecular Carcinogenesis, 1, 171-179, (1988) and Miller, D. R., Viaje, A., Aldaz, C.M., Conti, C.J. and Slaga, T.J., (1987), "Terminal Differentiation-Resistant Epidermal Cells in Mice Undergoing Two-stage Carcinogenesis", Cancer Res., 47: 1935-1940). However, they have undoubtedly undergone significant genetic and/or physiological alterations during their derivation. For example, they form benign papillomas upon skin grafting onto mice (Miller et al, Cancer Res., 47, 1935-1940, (1988)), are TGF-β resistant, and are likely to have suffered an activating ras mutation, which is a common event during chemical carcinogenesis of mouse skin (Balmain, A., Ramsden, M., Bowen, G. T., and Smith J., (1984), "Activation of the Mouse Cellular Harvey-ras Gene in Chemically Induced Benign Skin Papillomas", Nature, 307, 658-660). Thus, they are a sensitive indicator cell line to detect additional changes that can drive a minimally-transformed keratinocyte to frank tumorigenicity. However, the tumorigenic conversion induced by papillomavirus E5 genes in p117 keratinocytes is not a peculiarity of this cell line, since applicants have seen similar effects in a second, spontaneously derived murine keratinocyte cell line (pam 212 cells). The possibility exists that the E5 protein may not be sufficient for immortalization and transformation in vivo, but

rather that it may elicit its effects in cooperation with other viral genes, particularly the E6 and E7 genes. The finding that the E5 gene is only infrequently present in HPV-associated human carcinomas is consistent with the notion that it may act at an early stage during human tumorigenesis and that it is not required later on.

Papillomavirus E5 genes can evidently activate biochemical pathways in keratinocytes to cause tumorigenic transformation. The tumorigenic potential conferred on keratinocytes by HPV E5 expression may well be accompanied by alterations in the growth properties of the cells in culture or by biochemical differences compared to the parental cells without E5. Such in vitro differences are often present in tumorigenic cells and in the case of keratinocytes may include the expression or repression of particular genes or proteins, differences in other biochemical parameters such as enzymatic activities, or the ability or inability to grow in certain in vitro culture conditions. Thus the present invention has potential for use in screens other than for tumorgenicity. It has been proposed that the activated PDGF ß receptor is an important intracellular intermediate that transduces the transforming activity of the BPV E5 protein in fibroblasts. The keratinocytes described in the examples herein did not appear to contain PDGF ß receptors, indicating that this mechanism is not operative in this cell type, which is representative of the second natural host cell type of BPV. Biochemical analysis of E5-transformed keratinocytes may identify the signal transduction pathways activated in these cells.

Although it is possible that the HPV16 E5 gene may display weak transforming activity in fibroblasts under certain assay conditions (Vousden, K.H., Doniger, J., DiPaolo, J.A. and Lowry, D. R., (1988), "The E7 Open Reading Frame of Human Papillomavirus Type 16 Encodes a Transforming Gene", Oncogene Research, 3, 167-175), fibroblast transforming activity could not be documented using expression vectors, host cells, and transformation assays which readily detected the transforming activity of the BPV E5 gene. Thus, the BPV and the HPV16 E5 genes can both induce tumorigenic transformation of cultured murine epidermal keratinocytes, but only the viruses carrying the BPV E5 gene display significant transforming activity in fibroblasts. This difference parallels the different tissue tropism of the two papillomavirus types, with BPV inducing fibropapillomas (consisting of both mesenchymal and epithelial proliferative components) and HPV16 inducing papillomas (consisting exclusively of epithelial cells). Thus, the differential transforming activity of the E5 proteins may contribute to the tissue tropism of these virus types during natural infection. However, S.-L. Chen, and P. Mounts in "Transforming Activity of E5a Protein of Human Papillomavirus Type 6 in NIH3T3 and C127 Cells", J. Virol., 64, 3226-3233 (1990) have recently reported that the HPV6c E5a gene is able to induce morphologic transformation in NIH3T3 and C127 fibroblasts even though HPV6c normally induces purely epithelial lesions in humans. Thus, the ability of the HPV6c E5 gene to transform fibroblasts in culture does not signify fibrotropism of this virus in vivo. It is not known whether this

difference compared to the HPV 16 E5 gene reflects differences in the assay systems employed or intrinsic differences between the E5 genes of the two virus types. However, the predicted sequence of the HPV6c E5a protein is quite distinct from that of the HPV 16 E5 protein, and that HPV6c differs in many of its biological properties from HPV16.

The papillomaviruses have emerged as leading candidates for human tumor viruses and as useful model systems for determining the viral basis of cell transformation. It is becoming clear that these viruses have multiple transforming genes that may play relatively discrete roles in tumorigenesis. It is considered that the E5 proteins may play central roles in causing the pathogenic effects of the human as well as the fibropapillomaviruses.

Keratinocyte or epithelial cell lines that express human papillomavirus E5 gene according to the invention are useful for drug screens. Particularly, keratinocytes transformed by HPV16 E5 gene can be used to establish a screen to identify compounds that inhibit E5 action. Compounds can be tested for their ability to inhibit the properties of the cells that are due to the expression of the E5 protein. Such properties would include the ability of these cells to form tumors in animals, e.g., mice, as well as altered growth properties or altered biochemical characteristics that these cells may express in cell culture. Compounds identified in this manner may be useful as antiviral agents or as chemotherapeutic drugs active against tumors induced by human papillomaviruses.

Examples

Example 1: Cell Culture

C127, NIH3T3, ψcre, and PA317 cells and their derivatives were grown in Dulbecco's Modified Eagle's medium containing 10% fetal calf serum plus antibiotics (DME-10). pll7 cells were maintained in low calcium (0.05 mM) minimal essential medium supplemented with growth factors as described in Miller, D. R., Viaje, A., Aldaz, C. M., Conti, C. J. and Slaga, T. J., (1987), "Terminal Differentiation - Resistance Epidermal Cells in Mice Undergoing Two-Stage Carcinogenesis", Cancer Res., 47, 1935-1940. Cultures were supplemented with G418 as appropriate.

Example 2: Construction of Retroviral Vectors and Virus Stocks

Standard techniques were used to insert Xho I sites on both sides of the BPV E5 gene in pBPV-H11 (DiMaio, D., Treisman, R. H., and Maniatis, T. (1982), "A Bovine Papillomavirus Vector that Propagates as a Plasmid in Mouse and Bacterial cells, Proc. Natl. Acad. Sci (USA), 79, 4030-4034). Short Xho I fragments containing the E5 gene were then ligated into Sal I digested pLXSN-Sal, a derivative of the retroviral vector pLXSN (Miller, A. D. and Rusman, G. J., (1989), "Improved Retroviral Vectors for Gene Transfer and Expression", Biotechniques, 7,980-990) in which the unique Xho I site downstream of the viral left LTR was converted into a unique Sal I site. The nature and orientation of the inserts were confirmed by restriction mapping and DNA sequencing

and are summarized in Fig. 1 (the E5 coding region extends from BPV nucleotide (nt) # 3879 to 4010). pRV-BE5-L1M contains a single insert extending from BPV nt #3876 to 4022, pRV-BE5-L1D contains a head-to-tail tandem dimer of the same insert, pRV-BF5-L1M-FS contains a four base pair frameshift deletion three codons downstream of the E5 protein initiation codon in the monomer insert, and pRV-BE5-L3 contains BPV nt #3876 to 4072. A segment of HPV16 DNA from nt #3769 to 4151 was amplified by using the polymerase chain reaction and HPV16- specific primers containing Xho I sites (the HPV16 E5 coding region extends from nt # 3849 to 4098). After Xho I digestion, the amplified segment was inserted into Sal I-digested pLXSNSal to generate pRV-H16E5. Two independent clones with the gene in the correct orientation were isolated (pRV-H16E5-1 and pRV-H16E5-6), and the wild type DNA sequence of the entire HPV segment was confirmed for both isolates.

The calcium phosphate transfection technique was used to introduce intact plasmids containing the retrovirus vector into the ecotropic packing cell line, γcre (Danos, O. and Mulligan, R. C., (1988), "Safe and Efficient Generation of Recombinant Retroviruses and Amphotropic and Ecotropic Host Ranges", Proc. Natl. Acad. Sci USA, 85, 6460-6464). After being plated sparsely, colonies resistant to 600 μg/ml G418 were selected, and individual colonies were isolated and expanded into clonal cell lines. Cell lines producing high titer stocks of recombinant

retrovirus were identified by transduction of G418 resistance into fresh C127 cells. All γcre cell lines generated with the vectors containing the HPV16 E5 gene produced low titer virus stocks. Therefore, transient stocks generated by transfection of γcre cells with pRV-H16E5-1 and pRV-H16E5-6 were used to infect the amphotropic packaging cell line, PA317 (Miller, A.D. and Buttimore, C., (1986), "Redesign of Retrovirus Packaging Cell Lines to Avoid Recombination Leading to Helper Virus Production", Mol. Cell. Biol., 6, 2895-2902), and clonal PA317 cell lines producing high titer stocks of recombinant retroviruses were isolated and identified as above. Retroviruses are designated by the prefix RV-.

Example 3: Virus Infection and Transformation Assays

Virus stocks supplemented with 4μg/ml polybrene were used to infect subconfluent cultures of cells. For comparison of different virus stocks in any given cell line, the titer of virus (as determined by G418 resistant colony forming units) was adjusted so that infection was performed at a uniform multiplicity. After passage, infected cultures were incubated in media without drugs to select transformed foci or in media containing 600 μg/ml G418 (200 μg/ml G418 for pl17 cells and their derivatives). Individual G418-resistant colonies were picked and expanded into cell lines for analysis, or cells on plates containing at least 50 G418-resistant colonies were pooled

to establish pooled cell lines. For infection of p117 cells, virus stocks were prepared in the calcium-free medium used to propagate these cells.

Individual G418-resistant colonies or cell lines derived from such colonies were examined microscopically for characteristics of morphologic transformation including increased refractility, altered shape, and ability to overgrow the cell monolayer. Cell lines were tested for tumorigenicity by injecting 10^6 cells subcutaneously into 4-6 week old female Balb/c nude mice. Tumor formation was scored after three weeks (C127 derivatives) or four weeks (NIH3T3 and p117 derivatives). Individual tumors were excised, fixed in formalin, imbedded in paraffin, and stained with hematoxylin and eosin using standard techniques.

Example 4: Biochemical Analysis of Cell Lines

Total RNA was prepared from G418-resistant cell lines by using the guanidinium isothiocyanate method (Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J., (1980), "Isolation of Biologically Active Ribonucleic Acid From Sources Enriched in Ribonuclease", Biochemistry, 18,5294-5301). After electrophoresis on 1 % agarose gels containing formaldehyde and transfer to nitrocellulose, E5-specific sequences were detected by hybridization to ^{32}P-labelled fragments of HPV16 DNA (nt #

3751 to 4151) or BPV DNA (nt # 3881 to 4450), followed by autoradiography.

Example 5: Introducing Papillomavirus E5 Genes into Cells

To introduce papillomavirus E5 genes into cells, the coding region plus minimal 5' and 3' flanking sequences were cloned into a retrovirus vector under the transcriptional control of the viral LTR. As illustrated in Fig. 1, a series of BPVI E5 clones and an HPV16 E5 clone were constructed which were introduced into packaging cell lines to generate replication-defective, helper-free retroviral stocks. It was possible to monitor successful infection and stable integration of the provirus in infected cells because the viruses contained a neomycin resistance gene that conferred G418 resistance to cells harboring a transcriptionally active provirus.

Example 6: Activity of the E5 Genes in Fibroblasts

NIH3T3 fibroblasts and C127 cells were used for the analysis of the E5 genes in murine fibroblast cell lines. Most of the genetic and biological analysis of the BPV E5 protein has been performed in C127 cells, a flat, non-tumorigenic cell line derived from a murine mammary carcinoma (Dvoretzky, I,. Shober, R., Chattopadhy, S.K., and Lowy, D. R., (1980), "A Quantitative in vitro Focus Forming Assay for Bovine Papillomavirus", Virology, 103, 369-375) that appear to be representative of the fibroblastic host cell of the fibropapillomaviruses. C127 cells

were infected with approximately 1000 G418-resistance colony forming units of various recombinant retroviruses, passaged once, and maintained at confluence without biochemical selection. Recombinant virus stocks containing the BPV E5 gene induced the appearance of numerous transformed foci, whereas no foci appeared after infection with virus stocks of the vector without insert, the vector containing a BPV E5 gene with a frameshift mutation, or two independent isolates of viruses containing the HPV E5 gene (Table 1).

Table 1

TRANSFORMING ACTIVITY OF E5 RETROVIRUSES IN CULTURED FIBROBLASTS

			C127 CELLS	NIH3T3 CELLS
Virus	E5 gene	Foci	Morphologic Transformation[a]	Morphologic transformation[a]
RV-LXSN-Sal	none	0	0/20	0/20
RV-H16E5-1	HPV16	0	0/11	0/6
RV-H16E5-6	HPV16	0	0/8	0/6
RV-BE5-L1M	BPV	401	not done	
RV-BE5-L1M-FS	BPV(mutant)	0	0/20	
RV-BE5-L1D	BPV	339	20/20	
RV-BE5-L3	BPV	146	18/20	

[a]Number of cell lines appearing morphologically transformed over total number of cell lines examined. All cell lines were derived from individual G418-resistant colonies.

Similarly, the HPV16 E5 retroviruses did not induce foci in NIH 3T3 fibroblasts (data not shown). The results indicate that the cloned BPV insert contained sufficient genetic information to induce stable transformation of rodent fibroblasts, whereas the HPV E5 gene displayed no such activity.

In a less stringent assay for transformation, C127 and NIH3T3 cells were infected at low multiplicity with the E5

retroviruses, G418-resistant colonies and cell lines were generated, and the behavior of these cells was examined. Many of the colonies induced by viruses carrying the BPV E5 gene contained morphologically transformed cells, whereas infection with the control viruses or viruses containing the HPV E5 gene did not result in morphologic transformation (data not shown). Stable C127 and NIH3T3 cell lines were established from individual drug resistant colonies picked at random, and their morphology was assessed (Table 1). The great majority of colonies generated with the BPV E5 viruses gave rise to morphologically transformed cell lines, whereas none of the G418-resistant cell lines derived by infection with the control viruses or the HPV E5 viruses displayed morphologic alterations. The cell lines generated with the BPV E5 gene were also growth-transformed as judged by a number of additional criteria including anchorage independence, higher saturation density, shorter doubling time, and growth in low serum, whereas cell lines generated with the HPV16 E5 gene were indistinguishable from parental cells (data not shown).

To assess the tumorigenicity of fibroblasts transformed by the E5 gene, several of the G418 resistant cell lines were injected subcutaneously into nude mice. As shown in Table 2, rodent fibroblasts transformed by the BPV E5 gene induced tumor formation at the majority of injection sites, but cells infected with the vector alone did not display this activity.

Table 2

TUMORIGENICITY OF E5 RETROVIRUSES

		Tumors/injection sites[a]		
Virus	E5 gene	C127	NIH3T3	p117
RV-LXSN-Sal	none	0/12[b]	0/8	0/7
RV-BE5-L3	BPV	15/18[b]	not done	9/10[b]
RV-H16E5-1	HPV16	0/10	0/8	2/3
RV-H16E5-6	HPV16	0/10	0/20[c]	7/9

[a] A single pooled cell line was tested in each case, unless otherwise indicated.

[b] Aggregate data for two independently derived pooled cell lines.

[c] Aggregate data for one pooled cell line and two independent clonal cell lines.

These results establish that introduction of the BPV E5 coding region is sufficient to efficiently induce tumorigenic transformation in mouse C127 cells. In contrast, C127 and NIH3T3 cell lines established by infection with the HPV16 E5 retroviruses were not tumorigenic in multiple tests of both pooled and clonal cell lines established on several occasions with independent virus stocks (Table 2). Thus, in contrast to cells established with the BPV E5 retroviruses, fibroblasts infected with HPV E5 retroviruses did not display evidence of growth transformation.

Biochemical analysis of the transformed cell lines documented the expression of the E5 genes. Total RNA was prepared and analyzed for E5-specific sequences by Northern analysis. As shown in Fig. 2, cells infected with either the BPV or HPV E5-containing retroviruses expressed abundant E5 RNA that was absent from parental cells or G418-resistant cells established with the vector alone. In addition, the BPV E5 protein was present in cells infected with the E5 retrovirus (data not shown).

Example 7: Activity of E5 Genes in Keratinocytes

The activity of recombinant retroviruses expressing the E5 genes was also assessed in keratinocytes, one of the normal host cell types of BPVI and the sole normal host cell of HPV16. For

these studies mouse p117 cells were used, a non-tumorigenic keratinocyte cell line. In vivo tumorigenicity studies were relied upon to assess the ability of the E5 genes to transform these cells because morphologic transformation and focus formation are difficult to detect in cultured keratinocytes.

Pooled populations of G418-resistant p117 cells were established following infection with the control virus and viruses encoding the BPVI and HPV16 E5 genes. Assay of the culture media of G418 resistant p117 cells demonstrated the absence of viruses able to transduce G418 resistance (data not shown).

As shown in Fig. 3, BPVI and HPV 16 E5 RNA were readily detected in these cells. Injection of p117 cells infected with either the BPV or the HPV16 E5 viruses resulted in the appearance of tumors at most injection sites, whereas cells infected with the control virus were non-tumorigenic (Table 2). Parental p117 cells and p117 cells infected with a variety of other retrovirus vectors were also non-tumorigenic (Dotto, G.P., O'Connell, J., Patskan, G., Conti, C., Ariza, A. and Slaga. T. J., (1988), "Malignant Progression of Papilloma-Derived Keratinocytes: Differential Effects of the ras, neu, and p53 Oncogenes", Molecular Carcinogenesis, 1,171-179 and data not shown). These results were obtained with cell lines established from multiple independent infections for retroviruses transducing either the BPV or HPV16 E5 gene.

Histologic examination of these tumors revealed several features consistent with their being squamous cell tumors, including well-developed intercellular bridges, dyskeratotic cells, and horn pearls (Fig. 4). In general, the tumors induced by the BPV E5 viruses appeared to be squamous cell carcinomas. They grew more rapidly and to a larger size than the HPV16 E5 tumors, appearing histologically to be less differentiated and more invasive, infiltrating underlying connective tissue and muscle in five out of nine tumors examined.

These tumors also showed frequent mitotic figures and atypical nuclei.

The tumors induced by the HPV16 E5 retroviruses displayed features consistant with their being benign papillomas or well differentiated squamous cell carcinomas. They were well-delimited and circumscribed, and some of them were cystic with numerous papillas and keratinized centers. These differences may reflect biological differences between the two genes, or they may merely reflect experimental variability. In either case, these results establish that introduction of either the BPV or HPV16 E5 gene can induce tumorigenic transformation of the p117 keratinocyte cell line.

It will be appreciated that the instant specification is set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: DiMaio, Daniel C.
Dotto, Gian-Paolo

(ii) TITLE OF INVENTION: Keratinocyte or Epithelial Cell Line which Expresses Human Papillomavirus E5 Gene

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDANCE ADDRESS:

(A) ADDRESSEE: Frishauf, Holtz, Goodman, & Woodward, P.C.
(B) STREET: 600 Third Avenue
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10016-2088

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 5.5 inch, 360Kb
(B) COMPUTER: IBM PC/XT
(C) OPERATING SYSTEM: MS/DOS
(D) SOFTWARE: ASCII, converted using Wordperfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 07/701,413
(B) FILING DATE: 16-May-1991
(C) CLASSIFICATION:

(vii) ATTORNEY INFORMATION:

(A) NAME: Barth, Richard
(B) REGISTRATION NUMBER: 28,180
(C) REFERENCE/DOCKET NUMBER: 910304/RSB

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212)-972-1400
(B) TELEFAX: (212) 370-1622
(C) TELEX: 236268

(2) INFORMATION FOR SEQ ID NO. : 1:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 383 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: genomic DNA
(A) DESCRIPTION: Human papillomavirus type 16 E5 gene insert in retroviral vector.

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human papillomavirus type 16

(vii) IMMEDIATE SOURCE:
(B) CLONE: pRVH16E5

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Bubb, V.
McCance, D.J.
Schlegel, R.
(B) TITLE: DNA sequence of the HPV-16 E5 ORF and the structural conservation of its encoded protein.
(C) JOURNAL: Virology
(D) VOLUME: 163
(E) ISSUE: 1
(F) PAGES: 243-247
(G) DATE: MAR-1988

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Halbert, C.L.
Galloway, D.A.
(B) TITLE: Identification of the E5 open reading frame of human papillomavirus type 16
(C) JOURNAL: Journal of Virology
(D) VOLUME: 62
(E) ISSUE: 3
(F) PAGES: 1071-1075
(G) DATE: MAR-1988

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Seedorf, K.
Krammer, G.
Durst, M.
Suhai, S.
Rowekamp, W.
(B) TITLE: Human papillomavirus type 16 DNA sequence.
(C) JOURNAL: Virology
(D) VOLUME: 145
(E) ISSUE: 1
(F) PAGES: 181-185
(G) DATE: JAN-1985

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

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                     Met Thr Asn Leu Asp Thr Ala Ser Thr Thr
                      1               5                  10

TTA CTG GCG TGC TTT TTG CTT TGC TTT TGT GTG CTT TTG TGT GTC TGC     158
Leu Leu Ala Cys Phe Leu Leu Cys Phe Cys Val Leu Leu Cys Val Cys
                 15                  20                  25

CTA TTA ATA CGT CCG CTG CTT TTG TCT GTG TCT ACA TAC ACA TCA TTA     206
Leu Leu Ile Arg Pro Leu Leu Leu Ser Val Ser Thr Tyr Thr Ser Leu
             30                  35                  40

ATA ATA TTG GTA TTA CTA TTG TGG ATA ACA GCA GCC TCT GCG TTT AGG     254
Ile Ile Leu Val Leu Leu Leu Trp Ile Thr Ala Ala Ser Ala Phe Arg
         45                  50                  55

TGT TTT ATT GTA TAT ATT ATA TTT GTT TAT ATA CCA TTA TTT TTA ATA     302
Cys Phe Ile Val Tyr Ile Ile Phe Val Tyr Ile Pro Leu Phe Leu Ile
     60                  65                  70

CAT ACA CAT GCA CGC TTT TTA ATT ACA TAATGTATAT GTACATAATG           349
His Thr His Ala Arg Phe Leu Ile Thr
 75                  80

TAATTGTTAC ATATAATTGT TGTATACCAT AACT                               383
```

WHAT IS CLAIMED IS

1. A keratinocyte or epithelial cell line that expresses human papillomavirus E5 gene.

2. The cell line according to claim 1, wherein the human papillomavirus E5 gene is human papillomavirus type 16 E5 gene.

3. The cell line according to claim 2 wherein the cell line is a keratinocyte cell line.

4. The cell line according to claim 3, wherein the keratinocyte cell line is a murine kerotinocyte cell line.

5. The cell line according to claim 4, wherein the murine kerotinocyte cell line is p117.

6. The cell line according to claim 4, wherein the murine kerotinocyte cell line is Pam 212.

7. The cell line according to claim 1, wherein the human papillomavirus E5 gene is a human papillomavirus type E5 gene selected from the group consisting of type 1, type 2, type 6, type 11 and type 18.

8. The cell line according to claim 1, wherein the gene has the following sequence (SEQ ID No. 1):

```
AGTGAATGGC AACGTGACCA ATTTTTGTCT CAAGTTAAAA TACCAAAAAC TATTACAGTG   60

TCTACTGGAT TTATGTCTAT ATG ACA AAT CTT GAT ACT GCA TCC ACA ACA       110
                      Met Thr Asn Leu Asp Thr Ala Ser Thr Thr
                       1               5                  10

TTA CTG GCG TGC TTT TTG CTT TGC TTT TGT GTG CTT TTG TGT GTC TGC     158
Leu Leu Ala Cys Phe Leu Leu Cys Phe Cys Val Leu Leu Cys Val Cys
                   15                  20                  25

CTA TTA ATA CGT CCG CTG CTT TTG TCT GTG TCT ACA TAC ACA TCA TTA     206
Leu Leu Ile Arg Pro Leu Leu Leu Ser Val Ser Thr Tyr Thr Ser Leu
               30                  35                  40

ATA ATA TTG GTA TTA CTA TTG TGG ATA ACA GCA GCC TCT GCG TTT AGG     254
Ile Ile Leu Val Leu Leu Leu Trp Ile Thr Ala Ala Ser Ala Phe Arg
           45                  50                  55

TGT TTT ATT GTA TAT ATT ATA TTT GTT TAT ATA CCA TTA TTT TTA ATA     302
Cys Phe Ile Val Tyr Ile Ile Phe Val Tyr Ile Pro Leu Phe Leu Ile
       60                  65                  70

CAT AGA CAT GCA CGC TTT TTA ATT ACA TAATGTATAT GTACATAATG           349
His Thr His Ala Arg Phe Leu Ile Thr
   75                  80

TAATTGTTAC ATATAATTGT TGTATACCAT AACT                               383
```

9. A method for determining if a drug interferes with the property of cells that is due to the expression of the E5 protein, the method comprising

administering to an animal an effective amount of a keratinocyte or epithelial cell line that expresses human papillomavirus E5 gene,

administering to said animal a drug, and

determining if said drug interferes with the ability of the cells to display a property that is due to the expression of the E5 protein.

10. The method according to claim 9, wherein the human papillomavirus E5 gene is human papillomavirus type 16 E5 gene.

11. The method according to claim 10, wherein the cell line is a murine kerotinocyte cell line.

12. The method according to claim 11, wherein the cell line is p117.

13. The method according to claim 11, wherein the cell line is Pam 212.

14. The method according to claim 11, wherein the gene has the following sequence (SEQ ID NO. 1):

```
AGTGAATGGC AACGTGACCA ATTTTTGTCT CAAGTTAAAA TACCAAAAAC TATTACAGTG   60

TCTACTGGAT TTATGTCTAT ATG ACA AAT CTT GAT ACT GCA TCC ACA ACA       110
                      Met Thr Asn Leu Asp Thr Ala Ser Thr Thr
                       1               5                  10

TTA CTG GCG TGC TTT TTG CTT TGC TTT TGT GTG CTT TTG TGT GTC TGC     158
Leu Leu Ala Cys Phe Leu Leu Cys Phe Cys Val Leu Leu Cys Val Cys
                   15                  20                  25

CTA TTA ATA CGT CCG CTG CTT TTG TCT GTG TCT ACA TAC ACA TCA TTA     206
Leu Leu Ile Arg Pro Leu Leu Leu Ser Val Ser Thr Tyr Thr Ser Leu
               30                  35                  40

ATA ATA TTG GTA TTA CTA TTG TGG ATA ACA GCA GCC TCT GCG TTT AGG     254
Ile Ile Leu Val Leu Leu Leu Trp Ile Thr Ala Ala Ser Ala Phe Arg
           45                  50                  55

TGT TTT ATT GTA TAT ATT ATA TTT GTT TAT ATA CCA TTA TTT TTA ATA     302
Cys Phe Ile Val Tyr Ile Ile Phe Val Tyr Ile Pro Leu Phe Leu Ile
       60                  65                  70

CAT ACA CAT GCA CGC TTT TTA ATT ACA TAATGTATAT GTACATAATG           349
His Thr His Ala Arg Phe Leu Ile Thr
75                  80

TAATTGTTAC ATATAATTGT TGTATACCAT AACT                               383
```

15. The method according to claim 9, wherein the property is tumor formation.

16. The method according to claim 9, wherein the property is alteration in growth properties of the cells in culture.

17. The method according to claim 9, wherein the property is expression or repression of a gene or protein.

18. The method according to claim 9, wherein the property is a difference in a biochemical parameter.

19. The method according to claim 18, wherein the biochemical parameter is enzymatic activity.

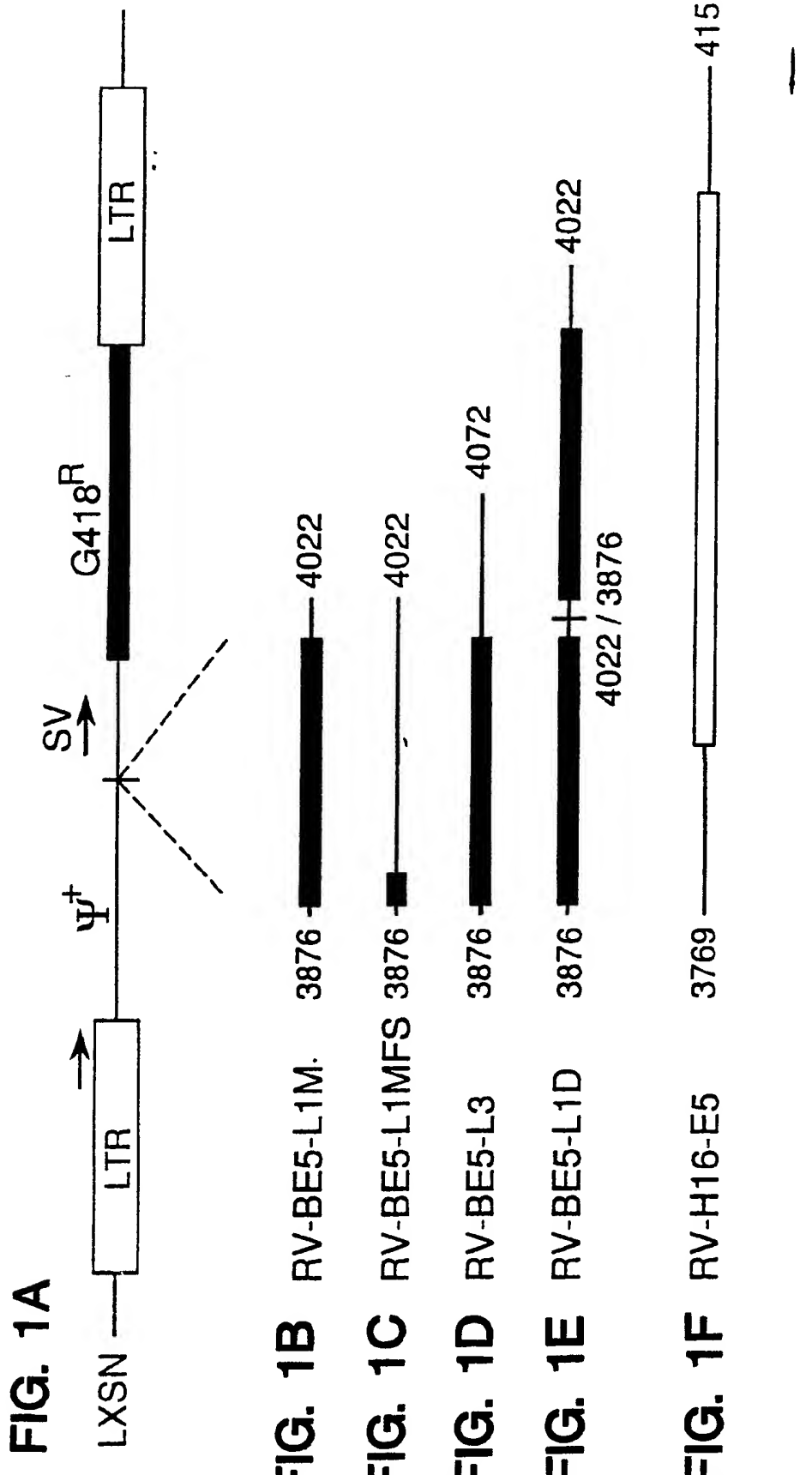

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1F

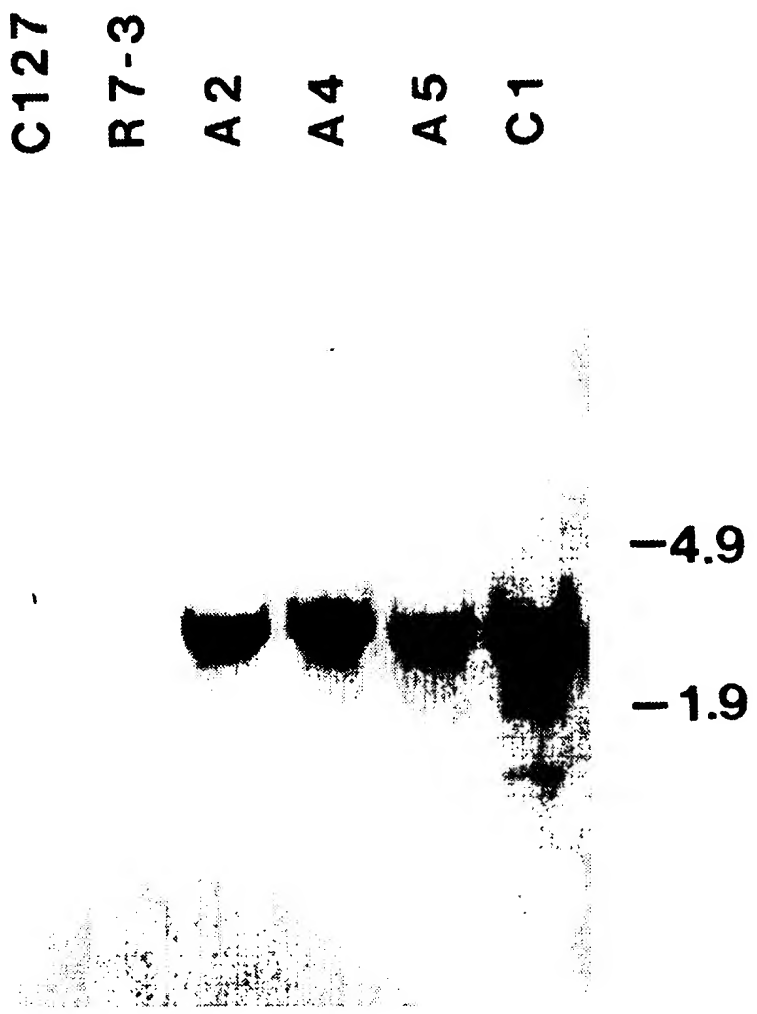

FIG. 2A

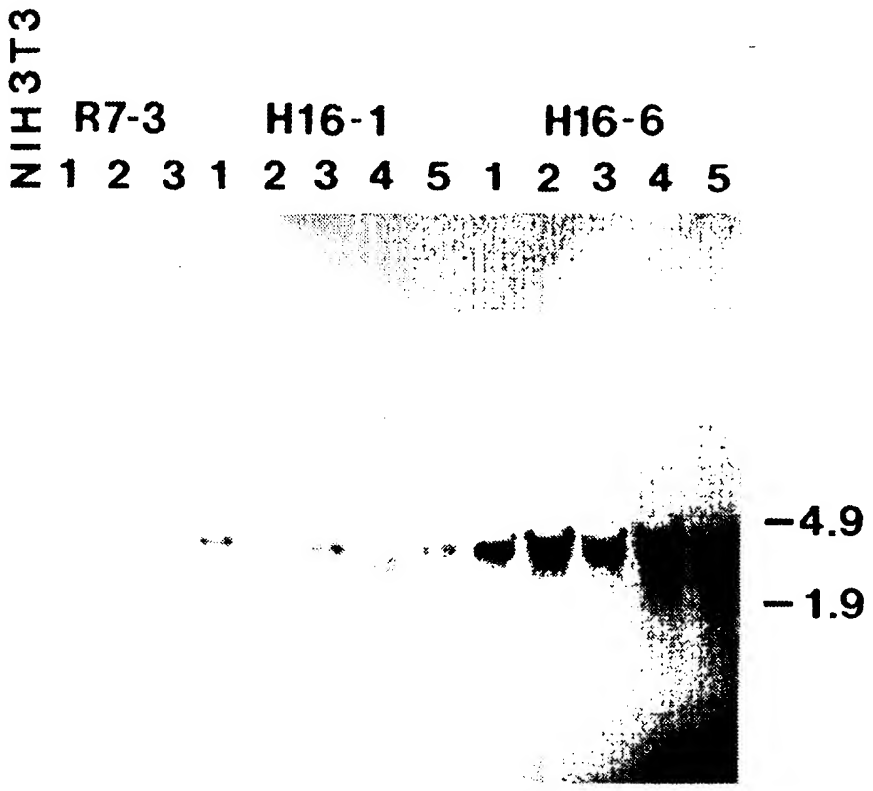

FIG. 2B

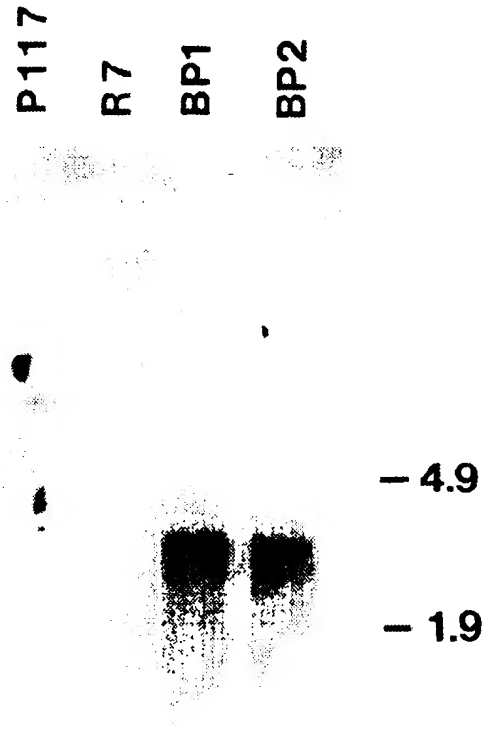

FIG. 3A

FIG. 3B

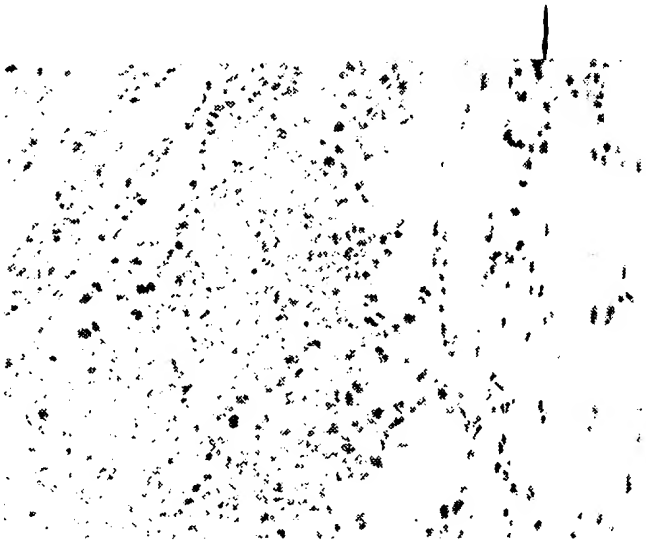

FIG. 4C

FIG. 4B

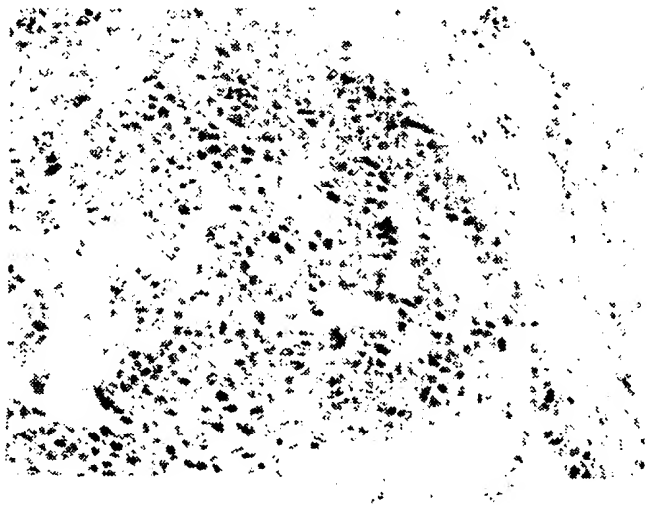

FIG. 4A

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08039

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 5/00, 7/04; C12Q 1/70, 1/00
US CL : 435/240.2, 236, 5, 7.23; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/240.2, 236, 5, 7.23; 536/27

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS; CAS, search subjects: human papillomavirus, keratinocyte

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	VIROLOGY, vol. 163, issued 1988, V. Bubb et al., "DNA Sequence of the HPV-16 E5 ORF and the Structural Conservation of Its Encoded Protein", pages 243-246, see entire document.	1-3/4-19
X/Y	J. VIROLOGY, vol. 64, No. 7, issued July 1990, Chen et al., "Transforming Activity of E5a Protein of Human Papillomavirus Type 6 in NIH 3T3 and C127 Cells," pages 3226-3233, see entire document.	1,7/2-6, 8-19
Y	VIROLOGY, vol. 175, issued April 1990, Parton et al., "Integrated HPV1 Genomes in a Human Keratinocytes Cell Line Can Be Transactivated by a SV40/BPV1 Recombinant Virus Which Expresses BPV1 E2 Proteins", pages 508-517, see entire documents.	1-19
Y	J. VIROLOGY, vol. 62, No. 3, issued March 1988, Halbert et al., "Identification of the E5 Open Reading Frame of Human Papillomavirus Type 16", pages 1071-1075, see entire document.	1-19

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
14 November 1991	06 DEC 1991
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	James Ketter

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	MOL. CELL. BIOL., vol. 10, No. 3, issued March 1990, E. Filvaroff et al., "Tyrosine Phosphorylation Is an Early and Specific Event Involved in Primary Keratinocyte Differentiation", pages 1164-1173, see especially page 1166.	6
Y	CHEMICAL ABSTRACTS, vol. 110, No. 19, issued 08 May 1989, G. P. Dotto et al., "Malignant Progression of Papilloma-derived Keratinocytes: Differentail Effects of the ras, neu, and p53 Oncogenes", see abstract no. 171036, Molecular Carcinog., 1, 171-179.	5

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[18] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[19]
Y	PROC. NATL. ACAD. SCI.,Vol. 83, issued 1986,DiMaio et al.,"Translation of Open Reading Frame E5 of Bovine Papillomavirus is Required for its Transforming Activity", pages 1797-1801, see entire document.	1-19

L11 ANSWER 7 OF 47 CAPLUS COPYRIGHT 2001 ACS
ACCESSION NUMBER: 1993:162460 CAPLUS
DOCUMENT NUMBER: 118:162460
TITLE: Keratinocyte or epithelial cell line expressing the human papillomavirus E5 gene
INVENTOR(S): Dimaio, Daniel C.; Dotto, Gian Paolo
PATENT ASSIGNEE(S): Yale University, USA
SOURCE: PCT Int. Appl., 46 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9220784	A1	19921126	WO 1991-US6039	19910823
W: CA, JP				
RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, NL, SE				
PRIORITY APPLN. INFO.:			US 1991-701413	19910516

AB The title **cell lines** are developed for use in **drug screening** to identify compds. that inhibit the action of the E5 transforming gene. A DNA fragment contg. the E5 gene from either bovine papillomavirus (BPV) or human papillomavirus type 16 (HPV16) was inserted into retroviral vector pLXSN, and plasmids contg. the vectors were transfected into ecotropic packing cell line .gamma.cre. The BPV gene cloned in .gamma.cre cells, was expressed in C127 murine mammary carcinoma fibroblasts as shown by E5 RNA and protein formation and foci of morphol. transformed cells in vitro, and induced tumors in nude mice injected with BPV-infected C127 cells or p117 mouse keratinocytes. Since .gamma.cre cells produced low titers of HPV16, this virus was cloned in amphotropic packaging cell line PA317; HPV16 gene E5 (sequence given) was expressed (E5 RNA formation) in both NIH 3T3 fibroblasts and p117 cells, and HPV16 was tumorigenic in p117 keratinocytes but not in NIH 3T3 fibroblasts.